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1. Lechmann et al. (Sminars in Liver disease, 2000, Vol. 2, pp. 211-226).
2. Lechner et al. (Philos. Trans. R. Soc. Lond. B. Bio Sci. 2000, Vol. 355, pp. 1085-1092).
3. Purcel (Hepatology 1997, Vol. 26, pp. 11S-14S).

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Why do cytotoxic T lymphocytes fail to eliminate hepatitis C virus? Lessons from studies using major histocompatibility complex class I peptide tetramers

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Hepatitis C virus (HCV) infection is a major public health problem, affecting an estimated 3% of the world's population, and over 10% in some countries. Infection in most cases becomes persistent, and can lead to hepatic inflammation, fibrosis and liver failure. The T lymphocyte reponse, in particular that mediated by cytotoxic T lymphocytes (CTLs), is likely to be involved in determining the outcome of infection, although its overall role is not clear. The use of major histocompatibility complex (MHC) class I peptide tetrameric complexes (tetramers) to study antiviral CTL responses has revolutionized our approach to the study of human infection. We have used a panel of MHC class I tetramers to analyse immune responses in HCV-infected individuals at various stages of disease. We find that the CTL response against HCV is vigorous in its early phases but dwindles over time both in terms of lymphocyte number and function. A number of potential explanations for this 'CTL failure' are discussed.

Keywords: hepatitis C virus; cytotoxic T lymphocyte; tetramer; immune escape; CD8

1. INTRODUCTION

Hepatitis C virus (HCV) is an important infection worldwide, including in Western countries, where spread through drug use and previously iatrogenically, through blood products or needles, has been significant (Cohen 1999). Although vertical spread is uncommon, it does occur and may lead to persistent infection in children of infected mothers (Heintges & Wands 1997), especially if the mother is human immunodeficiency virus (HIV) infected (Papaevangelou *et al.* 1998). After exposure, there is a range of possible clinical outcomes. At one extreme, patients may control virus below the level of detection in the blood (polymerase chain reaction (PCR)-negative) and suffer no long-term clinical sequelae. The majority of patients (*ca.* 85%) fail to clear virus (PCR-positive) and develop some degree of persistent hepatic inflammation. This in itself is quite variable and may represent only relatively low levels of mononuclear cell infiltration, with little or no fibrosis; in some patients, however, it may be much more aggressive, with progressive fibrosis over years, leading to cirrhosis and, ultimately, liver failure.

It is thought for a number of reasons that the cellular immune response against HCV, including that provided by cytotoxic T lymphocytes (CTLs), may play an important role in determining the subsequent course of the disease (Hirioshi *et al.* 1997; Koziel *et al.* 1995; Nelson *et al.* 1998; Rehermann *et al.* 1996*a*). CTLs are able to recognize an infected cell that displays viral peptides cleaved from nascent proteins and bound in the groove of class I major histocompatibility complex (MHC) molecules on the cell surface. They are able to emigrate into infected tissues and both destroy these infected cells (in principle before these cells have released new virions) and secrete a large array of antiviral and chemotactic factors. Thus they represent the ideal cells to contain infection in an inflamed 'peripheral' (i.e. non-lymphoid) organ. In animal models, most graphically in that of lymphocytic choriomeningitis virus in the mouse (LCMV), there is good experimental evidence that CTLs play a primary role in the immune control of acute infection, and also that they contribute to tissue damage, for example hepatic inflammation (Zinkernagel 1996; Zinkernagel *et al.* 1986). In human infections such as human immunodeficiency virus (HIV) and Epstein Barr virus (EBV), large populations of these cells can be observed, and their emergence may be temporally linked to control of viraemia (Callan *et al.* 1996, 1998; Koup *et al.* 1994). Finally, in a chimpanzee

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model of HCV, clearance after acute infection (in previously vaccinated animals) has been associated with broad and sustained CTL responses (Cooper *et al.* 1999).

CTLs do not act alone, and, even in the LCMV model where they are absolutely required for early viral control, they fail to suppress virus in the long term without CD4 helper cells or B cells secreting neutralizing antibody (Planz *et al.* 1997; Thomsen *et al.* 1996). In HCV, there is evidence that early broadly reactive CD4 responses may be associated with a favourable outcome (Diepolder *et al.* 1995; Gerlach *et al.* 1999; Missale *et al.* 1996). Whether this is due to a direct antiviral function or through provision of supportive cytokines for CTLs is not yet known. The role of neutralizing antibodies in HCV is controversial, as there is no generally available *in vitro* infection system with which to assess neutralization.

We have studied the immune response against HCV in several cohorts of patients, including those infected acutely or chronically (Lechner *et al.* 2000*a*,*b*), a set of long-term antibody-positive patients identified as blood donors and children born to infected mothers. To do this we have used a set of tetrameric MHC class I peptide complexes (tetramers) (Altman *et al.* 1996) to directly analyse CTL responses and determine the activation status and functional capacity of CTLs without *in vitro* manipulation. In some studies, we have compared this method with alternative measures of CTL activity such as ELIspot or cytotoxic T lymphocyte precursor (CTLp) measurement.

2. METHODS

(a) *Patients and samples*

All patients were human leucocyte antigen (HLA) typed by PCR and screened for the presence of HCV antibodies by ELISA (Abbott, Maidenhead, UK) and HCV RNA by nested reverse transcribed-polymerase chain reaction (RT-PCR). Only patients of the HLA-A2, B7 and B8 genotypes were included in these studies since the peptide epitopes of HCV and class I molecules for expression were best validated (Altman *et al.* 1996; Cerny *et al.* 1995; Koziel *et al.* 1995; Reid *et al.* 1996). Paediatric patients with hepatitis C were recruited from hospitals in New York (USA), adult patients from London and Oxford (UK) and healthy HCV-seropositive blood donors from Sydney (Australia).

Peripheral blood mononuclear cells (PBMCs) were obtained from patients by centrifugation over Lymphoprep (Nycomed, Oslo, Norway), followed by washing in RPMI 1640 (GIBCO BRL, Paisley, UK) and 10% foetal calf serum (FCS). PBMCs were frozen immediately and stored in liquid nitrogen until tetramer analysis was performed. Tetramer analyses of frozen sequential samples from one patient were performed wherever possible simultaneously after thawing.

(b) *Tetrameric MHC class I–peptide complexes*

Tetrameric peptide MHC class I complexes were made as described previously (Altman *et al.* 1996). Recombinant human β_2 microglobulin and the extracellular portion of the MHC class I heavy chain (containing the BirA recognition sequence in frame at its C-terminus) were expressed in *Escherichia coli* as insoluble aggregates which formed inclusion bodies. Purified inclusion bodies were solubilized in urea and monomeric HLA class I complexes refolded around peptide by dilution of denaturing conditions. The following HLA-A2 restricted peptides were used: hepatitis C virus NS3 1073-1081 (CINGVCWTV), NS3 1406-1415 (KLVALGINAV), NS4B 1807-1816 (LLFNIL GGWV) (Cerny *et al.* 1995; Koziel *et al.* 1995; Wong *et al.* 1998) and EBV lytic protein BMLF1 (GLCTLVAML) (Callan *et al.* 1996). HLA B7-restricted peptides derived from the core (GPRLGVRAT and DPRRRSRNL) and HLA B8-restricted peptides (HSKKKCDEL and LIRLKPTL) (Wong *et al.* 1998; D. Wong, personal communication) were also synthesized and refolded around recombinant molecules prepared in the same manner, together with controls using peptides derived from EBV (RPPIFIRRL and RAKFKQLL, respectively) (Callan *et al.* 1996). After buffer exchange, a specific lysine residue in the heavy chain C-terminal tag was biotinylated with BirA enzyme (Avidity, Denver, CO, USA). Monomeric complexes were purified by gel filtration and anion exchange chromatography. Tetrameric arrays of biotinylated peptide–MHC class I complexes were formed by the addition of phycoerythrin (PE)-labelled avidin (Extravidin, Sigma, St Louis, MO, USA) at a molar ratio of 4:1.

(c) *Flow cytometry*

Thawed PBMCs were washed three times in RPMI 1640 and 10% FCS and stained with tetrameric complexes and antibodies. The following monoclonal antibodies were used: anti-CD8-TriColor (Caltag, Burlingame, CA, USA), anti-HLA DR FITC (Dako, Cambridge, UK) and anti-CD38-APC (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). One million PBMCs were first incubated with tetrameric complexes for 15 min at 37 °C, then the antibodies were added and incubated for another 20 min on ice. The cells were washed with PBS and fixed with 0.5% formalin PBS. Samples were analysed on a FACScalibur flow cytometer, and the data were analysed using CELLQuest software (Becton-Dickinson).

The validation of the tetramers has been described elsewhere (Lechner *et al.* 2000*a*), and a similar approach has recently been published by an independent group (He *et al.* 1999). Staining was performed using clones of known specificity (provided by Andy Cerni and David Wong) to confirm binding activity. Prior experiments using these tetramers have given a lower limit of detection of *ca.* 0.02% of $CD8^+$ cells, in line with previous studies (Callan *et al.* 1998; Dunbar *et al.* 1998; Ogg *et al.* 1998).

(d) *CTL precursor measurement*

This was performed by limiting dilution assay, and activity tested against cells infected with recombinant vaccinia viruses (encoding HCV antigens from core, NS3, NS4 and NS5) as described previously (Borysiewicz *et al.* 1988; Carmichael *et al.* 1993).

3. RESULTS

(a) *Acute disease*

The immunological events surrounding acute infection with HCV have been poorly defined up until now, as patients rarely present at this early stage. Our tetramer studies of a cohort of acutely infected patients from Oxford, UK, Boston, MA, USA, Bari, Italy and Munich, Germany form the basis of separate submissions (Lechner *et al.* 2000*a*,*b*), but the principal findings are summarized as follows (see figure 1). Large, activated CTL responses may be evoked, in which up to 7% of CD8 lymphocytes are specific for a single epitope, although these may be transient in nature, and show rapid changes in surface phenotype and antigenic specificity. When the acute disease (hepatitis with abnormal liver function) subsides, CTL

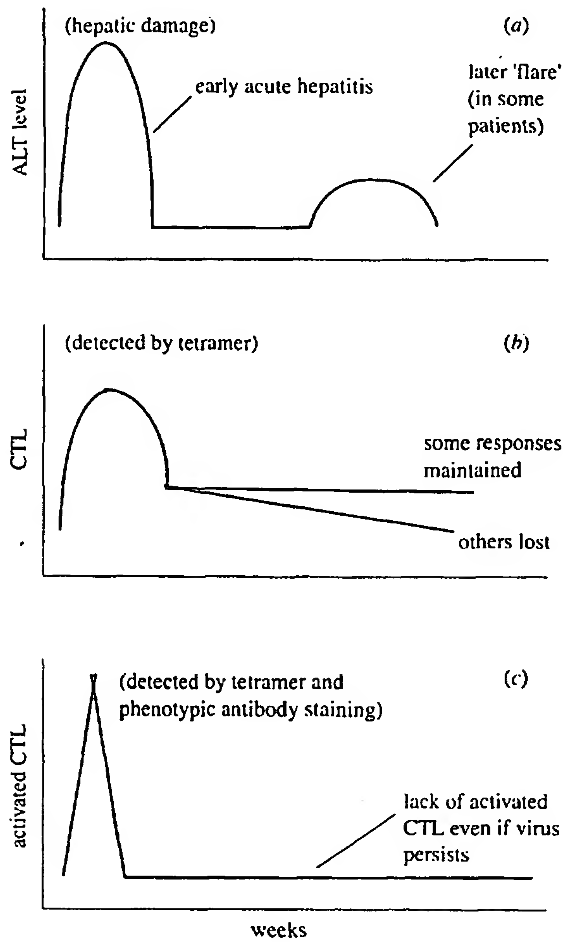

Figure 1. Schematic of CD8+ T-cell activity during and after acute disease. The events represented are a summary of different patients studied during and after acute HCV infection. (*a*) The acute rise in liver enzymes (notably alanine aminotransferase (ALT)) is seen. Accompanying this in (*b*) expansions of tetramer-positive CD8 T cells are observed, but in (*c*), it is also seen that the activated portion of these tetramer-positive populations is short-lived. These results are quite consistent regardless of the outcome of infection, or the individual epitope recognized.

responses may still evolve, although the cells detected are not the phenotypically active 'memory' phenotype. Each patient studied presents a slightly different picture and to date we have been unable to identify a definite correlation between particular forms or patterns of response and different clinical outcomes. Data from studies of CD4+ T-cell responses and from animal models suggest, however, that a broadly reactive and strong, early, sustained response is associated with clearance of HCV from blood (Cooper *et al.* 1999; Diepolder *et al.* 1995; Missale *et al.* 1996; Naumov 1999), although this raises the question of why such a response does not always emerge. In our best-studied patient, a very broad and vigorous CD4+- and CD8+-mediated response (to eight distinct epitopes) was observed, associated with rapid and sustained clearance of virus from the blood (Lechner *et al.* 2000*a*).

These results have changed our view of HCV infection, which has been assumed to be a relatively indolent infection that does not evoke significant immune responses (compared for example with EBV or HIV). In contrast, it appears that a substantial immune response may be mounted early, but that it commonly fails to eliminate virus and furthermore, does not remain active even in the face of continuing viral replication.

In the rest of this paper we discuss the results of investigation of different patient groups, to provide some perspective on what CTL responses are observed in HCV under different conditions after acute infection, using the new technologies available.

Table 1. *Clinical and immunological characteristics of paediatric cohort*

(This table represents the clinical features of the children studied. All children were born to HIV-HCV co-infected mothers except one, whose mother was seropositive only for HCV.)

group	patients	PCR	ALT	tetramer
infected (HIV−)	4	+[a]	normal	3/4
infected (HIV+)	3	+++[b]	elevated	1/3
exposed uninfected	2	−	normal	0/2

[a] PCR-positive, but low level (not quantifiable).
[b] PCR-positive with high levels of virus on quantitative assay.

(b) *Analysis of paediatric patients*

The adult studies in acute HCV disease indicate that the immune responses in early HCV are quite substantial, particularly in those who clear virus from the blood (figure 1). We were interested to observe, therefore, the response to HCV after mother-child transmission, since this might similarly represent acute exposure to the virus. Twelve patients were studied (of whom four were also HIV co-infected). These were split clinically into four groups: HCV infected + HIV co-infected, HCV infected alone, HCV exposed but uninfected (table 1), and a final group where the HCV status of the child was not yet established. In some patients, blood was available from at least two time-points, in order to analyse populations over time. Because of limitations in the size of blood samples, in some cases tetramers were tested in pools, and therefore the bulk anti-HCV response measured. In all cases the total percentage of tetramer-positive cells measured was low (< 0.5%), and in no case was a large, defined population of tetramer-positive cells seen on the flow cytometric assay plot. The findings may be summarized as follows.

(i) In those patients who were HIV co-infected, there were high levels of virus and high levels of alanine aminotransferase (ALT), reflecting hepatic injury. In the remaining patients, lower levels of virus, or intermittently positive PCRs, were associated with normal liver function.
(ii) HCV-tetramer-positive cells were detected in one out of three patients with high levels of virus, three out of four of those with low levels of virus, and none of those who had not been infected. The exact numbers and specificity of these responses require further confirmation.

Table 2. *Clinical and immunological characteristics of the adult blood donor cohort*

(Samples were scored positive for tetramer staining only if the tetramer-positive population was present as a clearly distinct cloud on the FACS plot (> 0.02% of CD8 cells) and if the result was reproducible on repeat testing. Samples were tested with the panel of tetramers listed in §2, as appropriate for their tissue type, and repeated tests performed depending on the availability of cells.)

		HLA genotype			tetramer	
subject	PCR	A	B	CTLp	HCV	EBV
A2	pos	3	7,35	66	–	+
A3	pos	32	44	24	–	...[a]
A6	neg	2,68	27,49	0	–	...
A7	pos	2,30	7,18	26	–	+
A8	pos	2,3	7,60	...[a]	–	+
A9	pos	2,29	44	...[a]	–	...[a]
A10	pos	2,32	55,62	5	–	+
A12	neg	2	8,44	0	–	+
A13	pos	2,3	44,51	...[a]	–	+
A15	pos	3	35	...[a]	–	...[a]
A16	pos	2,28	38,35	...[a]	+	+
A17	neg	2,31	49	0	–	–
A19	pos	1,68	8,44	8	–	...[a]
A20	pos	1,2	8	8	–	+
A22	pos	1	60	0	–	...[a]
A23	pos	2	41	...[a]	–	...[a]
A24	neg	2,3	7,61	0	–	+
A25	pos	3,32	7	95	–	...[a]
A26	pos	24	7,35	10	–	...[a]
A29	pos	2,3	14,55	...[a]	–	–
A30	neg	1,2	37,60	0	–	...[a]

[a] not tested; CTLp, number of CTL precursors per million PBMCs.

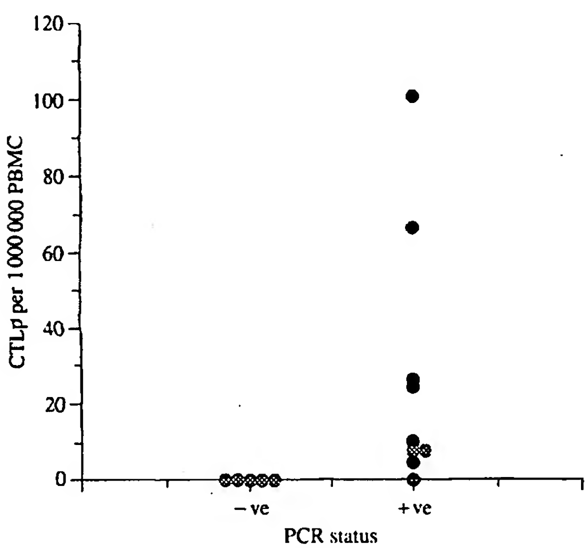

Figure 2. HCV-specific CTL precursor frequencies in PCR-positive and PCR-negative adult HCV seropositive patients. Each dot represents an HCV-specific CTLp measurement (see §2(d)) from an individual patient. The patients are described in table 2.

In acutely infected adult patients, large populations of tetramer-positive cells were observed during acute disease. However, these populations were short-lived, and dropped to very low levels after a few weeks, especially in those where infection was not cleared from blood. Since samples from children were taken when they were older than three months, these findings are therefore consistent with the data obtained in adults. That is, in those with chronic disease, there was significantly less tetramer staining seen than in those who had controlled virus in blood (Lechner *et al.* 2000*a*).

These findings suggest that CTLs play a role during infection of children of HCV-infected mothers, although more definitive data are required, particularly prospective studies, and studies of the very earliest stages of infection. The role of HIV co-infection in disease progression clearly warrants further analysis.

(c) *CTL responses in adult patients*

We investigated the CTL responses in established disease in a separate set of adult patients who were identified during a screening process at the time of blood donation. These were divided clinically into two groups those with persistent infection as determined by RT-PCR (PCR-positive) and those who had spontaneously cleared the virus below the limit of detection in blood (PCR-negative) (table 2). A set of 14 of these were available for analysis of CTLp and/or subsequent tetramer staining on frozen samples (nine PCR-positive, five PCR-negative). The results from the CTLp analysis are shown in figure 2; interestingly, CTLp levels were below the threshold of detection in all PCR-negative patients. Patients with persistent infection had detectable CTLp, at levels up to 1 in 10 000 PBMCs, with an average level of 27 per 1 000 000 PBMCs. These levels are consistent with previous studies (Hirioshi *et al.* 1997; Rehermann *et al.*

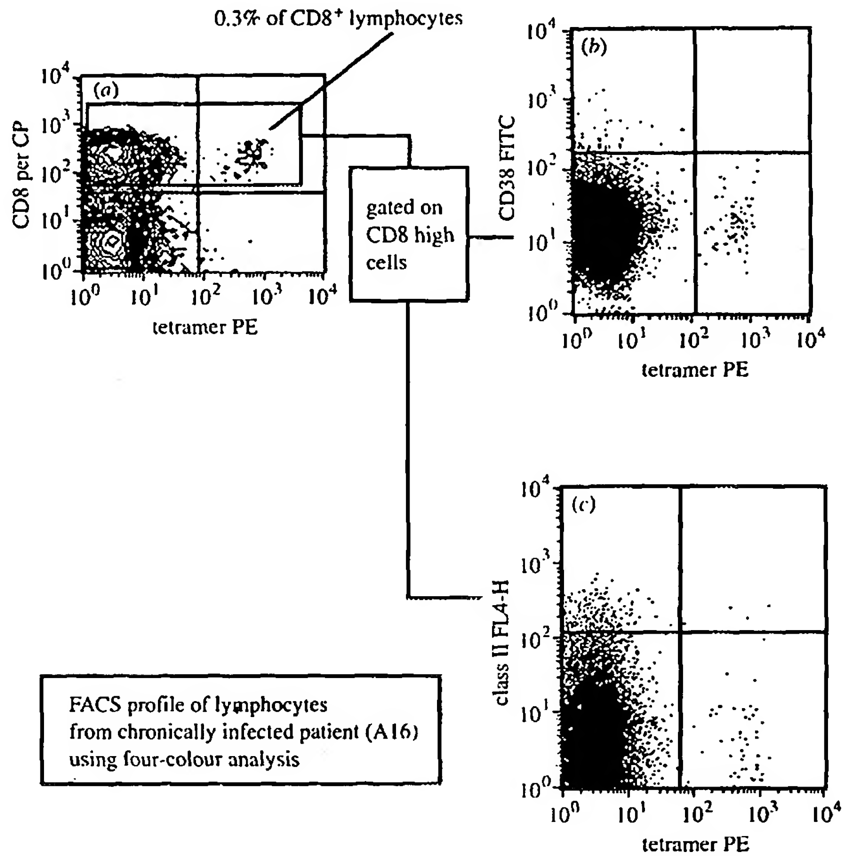

Figure 3. CTLs in adult HCV-seropositive patient A16. (*a*) Tetramer staining using HLA-A2 tetramer specific for epitope NS3 1073-81 as described in §2. A distinct population of tetramer-positive cells appears as a cloud in the right upper quadrant. (*b*, *c*) Phenotype of CTLs identified that stain with tetramer specific for NS3 1073-81. The CD8+ lymphocytes are selected from (*a*) and further analysed for the expression of activation markers CD38 and HLA-DR. The staining of the tetramer-positive cells is low for both of these markers, indicative of a quiescent or 'resting memory' phenotype.

1996*a*), although the complete lack of detectable CTLp in PCR-negative seropositive patients has not, to our knowledge, been described previously. The difference between the two groups (0/5 versus 8/9 positive responses) is not statistically significant ($p=0.076$ by Fisher's exact test). Much larger studies, screening for CTLs specific to multiple HCV epitopes, are required to address further the relationship between CTL frequency and viral persistence.

In the case of the CTL detected by tetramers, the results are less clear-cut, and in general very low levels of tetramer-positive cells were found in both groups ($<0.05\%$) without clear and distinct populations of tetramer-positive cells. Similar low-level reactivity or non-reactivity has been observed both by ourselves and others (He *et al.* 1999; Lechner *et al.* 2000*a*). We have found that screening with a panel of tetramers containing HLA-A2-restricted epitopes does not reveal large populations of CTLs that are not detectable by other means. Indeed, in this set of patients there were several examples where CTLp measurements were positive, but tetramers negative. The potential reasons for this are discussed below.

There was one exception to the general lack of tetramer-positive cells found—a seropositive, PCR-positive patient (A16), with levels of ALT just above normal (representing mild hepatic inflammation), and an unremarkable, chronic clinical course. The tetramer staining data from this patient are shown in figure 3. This figure shows a distinct cloud of A2-restricted NS3 1073-specific cells (figure 3*a*). These cells were low in surface expression of CD38 and HLA-DR (figure 3*b*,*c*). They also displayed classical memory markers such as CD62L low- and CD45RO high-staining (data not shown). In other words, this was a memory population with low levels of activation despite high levels of virus. In other studies we have performed in separate populations of patients, a similar low-activation phenotype has also been detected (Lechner *et al.* 2000*a*,*b*). The explanation for this isolated, large, 'resting' CTL population in this individual is not clear, but the functional status of the cells and the effect of variation within the target epitope are currently under investigation.

(d) *Further studies of activation status*

One potential explanation for the general lack of reactivity seen in chronic disease is that the patients' responses may have shifted on to new epitopes or may react to variant epitopes, which would not be detected by tetramer staining, or indeed conventional assays that rely

on known epitope sequences. To overcome this problem, we attempted to measure the overall levels of CD8⁺ activity by analysis of the CD38⁺/ HLA-DR⁺ double-positive subset. These markers have been found to reflect the presence of an antiviral, activated T-cell population in HIV and CMV (Belles-Isles *et al.* 1998; Giorgi *et al.* 1993; Ho *et al.* 1993) and their frequency varies with changes in viral load (Kaufmann *et al.* 1999). Interestingly, in a separate group of 18 HCV persistently seropositive patients, the levels of activated CTLs as judged by these activation markers were not elevated compared with controls from the literature (Belles-Isles *et al.* 1998; Giorgi *et al.* 1993), nor did they relate to either viral load or ALT levels. This was consistent with two possibilities: either only a small proportion of PBMCs was devoted to HCV surveillance or, as in the case A16 illustrated, there was low expression of these two activation markers. In those cases of HCV where we have been able to follow CTL through from acute disease, CD38 and HLA-DR expression have been rapidly lost and never regained, regardless of the presence of virus; so we favour the latter explanation.

4. CONCLUSIONS

This study confirms the adaptability of the tetramer technology for the analysis of CTL responses in a variety of situations, including children, where the sample size is often limiting. HCV is particularly difficult to study because of the known lack of immunodominance, the problems of variability both between viral strains and within individual patients over time, and because of the overall low levels of CTLs compared with many other antiviral responses (Rehermann *et al.* 1996*a*,*b*).

In the case of the adult HCV studies, already a good deal has been addressed by using conventional culture techniques in patients with established infection (Koziel *et al.* 1995; Rehermann *et al.* 1996*a*,*b*) to determine CTLp levels and specificity, although there has been no clear association with disease state. Our results in adult patients were surprising in that very low levels of CTLp in the PCR-negative group were obtained. This is contrary to the HIV experience in natural infection, where high levels of CTLs (by tetramer or conventional methods) are associated with low levels of virus (Ogg *et al.* 1998), although consistent with the experience in treated HIV disease, where the CTL level and activation state may reflect the viral load (Kaufmann *et al.* 1999). Indirectly, it has been argued that effective treatment relies on high levels of CTLs, as predicted from higher levels of ALT and lower viral load (Neumann *et al.* 1998). Further studies—especially those in which the complete spectrum of CTL responses against all epitopes is taken into account—are required to establish these associations more firmly.

Our studies with tetramers in the group of Australian blood donors reflect our experience with non-acute patients, with one exception. In general, we have found very low levels of tetramer-positive cells, which, when present, stain weakly for activation markers (except during acute disease). However, we have found slightly higher levels of CTLs in those with no detectable virus in serum (Lechner *et al.* 2000*a*,*b*). However, large numbers of patients are needed to confirm these results, given the generally low level of reactivity in these tests.

Why are the levels of tetramer-positive cells so low, except during the acute phase? First, there are a number of potential technical explanations.

(i) There is compartmentalization of immune responses in the liver (Minutello *et al.* 1993). To date we have found similar low levels of CTLs by tetramers in a series of cirrhotic liver samples compared with blood and lymph nodes from the same patients (Valiante *et al.* 2000). Although we cannot say for certain what is the case in non-cirrhotic livers, this observation makes this explanation less likely. Larger numbers of patients, especially those with documented tetramer-positive populations in the blood, are required to answer this question.

(ii) The patients are responding to different epitopes. Although this is still formally possible, as the selection of epitopes in HCV is very broad, this appears to be unlikely for the following reason. In acute disease, we have found, using the same tetramers, a high rate of positivity among infected patients (over 50%). Using a set of tetramers extending to nine epitopes (including the HLA B7- and B8-restricted epitopes), we have not seen a similar response in over 60 non-acute patients. If responses are present to these epitopes they are generally at a low level, with the exception of A16, illustrated here. Studies using sensitive and broad-based techniques, but which do not rely on interferon-γ secretion (which may be impaired), are required to answer this question completely.

(iii) T-cell receptors are downregulated, reducing tetramer binding. This has been observed during acute disease, but not to an extent that eliminates binding altogether. This has not been found to be a problem in other infections such as HIV. A specific effect of HCV cannot, however, be excluded.

If the levels of HCV-specific CTLs in chronic infection are truly low, as opposed to artefactually low, there are also a number of potential explanations. Antigenic variation leading to loss of CTL recognition is a possibility and has already been observed in HCV (Chang *et al.* 1997; Giuggio *et al.* 1998; Weiner *et al.* 1995). Such escape mutants have also been shown to act as antagonists (Rehermann *et al.* 1996*a*), and in doing so may adversely affect both the ongoing immune response and the ability of new clones of CTLs to expand *in vivo* (Klenerman *et al.* 1996; Klenerman & Zinkernagel 1998). HCV infection does not cause generalized immune suppression clinically, and an immune response against other co-infecting viruses such as HIV may be sustained. It may be that there is exhaustion or specific dysregulation of HCV-directed CTLs, in particular downregulation of cytokine secretion capacity (Lechner *et al.* 2000*a*). Finally, the role of the liver as a specific 'tolerogenic' environment must not be overlooked.

What then is the role of the CTLs in HCV infection—given that they are often so hard to find? Our current studies suggest that CTLs play an active role early in disease, presumably both through lysis of infected cells and secretion of antiviral cytokines. They may contribute

to clearance or control of virus, and also possibly to the acute hepatitic illness. Beyond this point, they may continue to play an active suppressive role in those who control virus, but in those in whom virus persists, their importance is unclear. They appear to exist at low levels (if at all) and to be unresponsive to antigen. In such patients it may be that small numbers are involved at the initiation of a cascade of liver inflammatory cells, of which they form only a small proportion. Exactly what proportion and what activation state these cells are in within the liver remains to be determined.

The study of HCV has reached an exciting stage—we now understand a good deal more of the events surrounding acute disease and some of the dynamic processes surrounding the first weeks of infection. The reasons why in the majority of patients the virus persists and why the immune response fails are still unclear. The ability to track CTL and, soon, CD4+ populations directly *ex vivo* and follow not only their number but their phenotype and function, is giving us new insight into the pathogenesis of this complex disease.

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